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Abstract: The two subunits of the feline immunodeficiency virus (FIV) reverse transcriptase (RT) were cloned and functionally expressed in Escherichia coli. The recombinant proteins are enzymatically active as homodimers (p66 and p51) as well as a heterodimer p66/p51. The biochemical properties of the FIV RT are very similar to those of the counterpart of the human immunodeficiency virus type 1 in being an RNA-dependent and DNA-dependent DNA polymerase. When a double-stranded DNA containing a small gap of 26 nucleotides was tested, we found a new activity of the FIV RT p66/p51 heterodimer–the cat viral enzyme could perform strand displacement DNA synthesis of approximately 300 bases. The FIV RT homodimer p66 alone could carry out limited strand displacement DNA synthesis, but this activity was stimulated by the p51 subunit at a molar ratio of one molecule of p66 to five molecules of p51. On the other hand, the homodimeric p51 itself was unable to fill a small gap of 26 nucleotides in a double-stranded DNA substrate and was not active by itself in strand displacement DNA synthesis. These data are in agreement with an earlier finding of strand displacement DNA synthesis by human immunodeficiency virus type 1 RT (M. Hottiger, V.N. Podust, R.L. Thimmig, C.S. McHenry, and U. Hübscher. J. Biol. Chem. 269:986-991, 1994). Our data therefore suggest a general and important function of lentiviral p51 subunits in strand displacement DNA synthesis which appears to be required in later stages of the lentiviral replication cycle, when DNA-dependent DNA synthesis occurs on double-stranded DNA.

DOI: https://doi.org/10.1128/JVI.69.10.6273-6279.1995

JOURNAL OF VIROLOGY, Oct. 1995, p. 6273–6279 Vol. 69, No. 10
0022-538X/95/$04.00+0

Feline Immunodeficiency Virus Reverse Transcriptase: Expression, Functional Characterization, and Reconstitution of the 66- and 51-Kilodalton Subunits

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Received 19 April 1995/Accepted 14 July 1995

The two subunits of the feline immunodeficiency virus (FIV) reverse transcriptase (RT) were cloned and functionally expressed in *Escherichia coli*. The recombinant proteins are enzymatically active as homodimers (p66 and p51) as well as a heterodimer p66/p51. The biochemical properties of the FIV RT are very similar to those of the counterpart of the human immunodeficiency virus type 1 in being an RNA-dependent and DNA-dependent DNA polymerase. When a double-stranded DNA containing a small gap of 26 nucleotides was tested, we found a new activity of the FIV RT p66/p51 heterodimer—the cat viral enzyme could perform strand displacement DNA synthesis of approximately 300 bases. The FIV RT homodimer p66 alone could carry out limited strand displacement DNA synthesis, but this activity was stimulated by the p51 subunit at a molar ratio of one molecule of p66 to five molecules of p51. On the other hand, the homodimeric p51 itself was unable to fill a small gap of 26 nucleotides in a double-stranded DNA substrate and was not active by itself in strand displacement DNA synthesis. These data are in agreement with an earlier finding of strand displacement DNA synthesis by human immunodeficiency virus type 1 RT (M. Hottiger, V. N. Podust, R. L. Thimmig, C. S. McHenry, and U. Hübscher. J. Biol. Chem. 269:986–991, 1994). Our data therefore suggest a general and important function of lentiviral p51 subunits in strand displacement DNA synthesis which appears to be required in later stages of the lentiviral replication cycle, when DNA-dependent DNA synthesis occurs on double-stranded DNA.

Feline immunodeficiency virus (FIV) belongs to the lentiviruses, which cause a progressive disruption of the host's immune functions (for review, see reference 2). Like human immunodeficiency virus (HIV), FIV shows a tropism for T lymphocytes and was early proposed to serve as a model for HIV studies (25). The genome organization is very similar to those of HIV, simian immunodeficiency virus, visna virus, and equine infectious anemia virus (21, 24).

FIV reverse transcriptase (RT) consists of two polypeptides with common N termini, a 66-kDa subunit (p66), and a 51-kDa subunit (p51); both subunits are present in equimolar amounts (ratio of 1:1) (22, 23). FIV RT p51 is generated by cleavage of the RNase H domain (p15) at the C terminus of FIV RT p66 by the virus-encoded protease. Sequence comparisons between HIV-1 RT and FIV RT reveal 63% identity at the nucleotide level and 48% identity and 67% similarity at the amino acid level, respectively.

RT is responsible for the conversion of genomic single-stranded RNA into double-stranded DNA. During this reverse transcription process, RNA-dependent as well as DNA-dependent DNA synthesis occurs (minus- and plus-strand DNA synthesis; for details, see references 15 and 37). Biochemical (5, 13, 33) and electron microscopic (14) data led to the generation of a model with multiple initiation events during plus-strand synthesis with a hypothesized partial strand displacement of already synthesized downstream plus strands by those originating upstream (6). A last step in reverse transcription is the extension of the 3′ terminus of the minus-strand DNA and generation of the long terminal repeat (LTR) with a linear duplex DNA as the end product (8). In FIV, the LTR region is 361 bp (2); in HIV-1, it is 636 bp (28). The ability of retroviral RTs to promote strand displacement DNA synthesis was shown in previous work (12, 20, 38).

In this work, we present the cloning, functional expression, and purification of the two enzymatically active FIV RT subunits. Furthermore, we show a new activity of the FIV RT heterodimer p66/p51. It possesses a strand displacement DNA synthesis activity, which could also be carried out in limited amounts by the FIV RT p66 homodimer alone. The FIV RT p51 homodimer, on the other hand, was unable to replicate extensively on natural DNA templates but could enhance the rate and extent of strand displacement DNA synthesis activity of FIV RT p66. Our data underline the similarity between FIV RT and its HIV-1 counterpart and suggest a general and important function of the p51 subunit of lentiviruses in strand displacement DNA synthesis.

MATERIALS AND METHODS

Chemicals. Radioactively labelled nucleotides were purchased from Amersham. All other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Buffers. The following buffers were used: buffer A, 50 mM Tris-HCl (pH 7.5), 6% (vol/vol) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride; buffer B, 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4 (pH 7.3); buffer C, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 2.5 mM $CaCl_2$; buffer D, 50 mM Tris-HCl (pH 7.5), 50% (vol/vol) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride; and buffer E, 50 mM Tris-HCl (pH 7.5), 50% (vol/vol) formamide, 20 mM EDTA, 0.03% (wt/vol) xylene cyanol, and 0.03% (wt/vol) bromphenol blue.

PCR. Genomic DNA of FL-4 cells permanently infected with FIV Petaluma (40) was extracted according to the method in reference 1 and was used as a template for amplification of the RT-encoding region of the *pol* gene. Sequence

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comparison with HIV-1 RT was used to find the putative cleavage sites of the protease in the *pol* precursor polyprotein. The region chosen for amplification spanned nucleotides (nt) 2336 to 3661 for p51 and nt 2336 to 4012 for p66 (32). The following oligonucleotides were designed: MA1 (5′ primer; 5′-CCGCCAG CGAATTCATCGATATTTCTGATAAGATTCCAGTAG-3′), MA2 (3′ primer; 5′-CTGACGGCGAATTCTCGAG**TTA**CCATGTTTCTGCTCCTGG-3′), and MA3 (3′ primer; (5′-GAGACCGGGAATTCTCGAG**TTA**CCCTTCTATT ATCATCATTG-3′); sequences complementary to FIV Petaluma are underlined, the newly introduced *Eco*RI, *Cla*I, and *Xho*I restriction sites are double underlined, and the added stop codon is set in boldface. Primer pairs MA1/MA2 and MA1/MA3 were used to amplify the p51 and p66 sequences, respectively. The PCR mixture contained in a final volume of 20 μl: 0.5 μg of DNA, 0.25 μM (each) primer, 2.5 mM $MgCl_2$, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (vol/vol) Triton X-100, 0.25 mM (each) four deoxynucleoside triphosphates (dNTPs), and 0.5 U of Vent DNA polymerase (New England Biolabs). The steps for PCR were 94°C for 1 min, 60°C for 1 min, and 73°C for 2 min. After 35 cycles, a small fraction was loaded for analysis on an agarose gel, and if the correct band was present, the product was purified by phenol-chloroform extraction and sequenced.

Construction of GST fusion proteins. The purified fragments were digested with *Eco*RI and ligated to the modified expression plasmid pGEX-2T digested with *Eco*RI, thus generating pGEX-FIV-RTp51 (pMA123) and pGEX-FIV-RTp66 (pMA127). pGEX-2T (Pharmacia) has been modified to encode a specific phosphorylation site for bovine heart muscle kinase (HMK) (3). The construction of plasmids for functional expression of HIV-1 RT p66 and p51 as glutathione *S*-transferase (GST) fusion proteins was described in reference 11. DNA sequencing was performed by using a dideoxy sequencing kit (U.S. Biochemical Corp.).

Enzymes and proteins. The technique for purification of the GST fusion proteins was based on the method described in reference 31. Expression of the GST-RT fusion proteins in the *Escherichia coli* JM109 was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and cells were grown at 37°C for 3 h (culture volume, 0.5 liter). The bacterial pellet was resuspended in 10 ml of buffer A, the cells were broken by a French press, and the lysate supernatant was incubated with 1 ml of glutathione agarose (GSH) beads (Pharmacia) for 12 h. After being washed with buffer B, the beads were resuspended in 1 ml of buffer C and digested with thrombin (100 U) overnight at 4°C. The suspension was centrifuged for 5 min at 4,000 × *g*, and the supernatant was diluted in buffer A and loaded on a fast protein liquid chromatography Mono S column equilibrated in buffer A. The FIV RT proteins were eluted from the column with a 0 to 0.5 M KCl gradient in buffer A. Fractions containing RT activity eluted at 250 mM KCl, were pooled, dialyzed against buffer D, and stored at −20°C. HIV-1 RT heterodimer p66/p51 was overexpressed in *E. coli* (gift of L. Loeb, Joseph Gottstein Memorial Cancer Research Laboratory, University of Washington) and purified as described in reference 12. Calf thymus DNA polymerase α was isolated according to the method in reference 27. Thrombin protease was purchased from Sigma (Buchs, Switzerland), and the restriction enzymes were obtained from Boehringer Mannheim or New England Biolabs.

Nucleic acid substrates. The homopolymers poly(rA) and poly(dA) (Pharmacia; concentrations were determined according to the manufacturer's protocol) were mixed at a weight ratio of 10:1 with the oligomer $oligo(dT)_{15–18}$ (Pharmacia) in 20 mM Tris-HCl (pH 8) containing 20 mM NaCl and 1 mM EDTA, and then the mixture was heated at 75°C for 10 min with subsequent slow cooling to room temperature.

A gapped circular DNA substrate was prepared as follows: Positive-strand and replicative form I DNAs of plasmid Bluescript SK(+) were prepared according to the supplier's instructions (Stratagene). Replicative form I DNA was digested with *Eco*RI and *Xba*I, and the ends of the vector were filled by Klenow DNA polymerase. The religated vector with the restored *Eco*RI site was linearized by *Eco*RI digestion and annealed to the circular, positive strand of Bluescript SK(+) in 60 mM Tris-HCl (pH 8)–0.1 M NaCl–10 mM EDTA for 2 min at 100°C, 15 min at 65°C, and 30 min at room temperature. The mixture was loaded on a 1% agarose gel in 1× TAE (30), and the DNA band corresponding to the circular, double-stranded plasmid with a 26-nt gap was eluted from the gel slice and purified by Gene Clean (Bio 101).

Enzymatic assays. (i) DNA polymerases. The RNA-dependent DNA polymerase activity of RT was determined in a mixture with final volume of 25 μl containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM [^{3}H]dTTP (300 cpm/pmol), 0.5 μg of $poly(rA)$-$oligo(dT)_{15–18}$ (base ratio of 10:1), 0.05% (vol/vol) Triton X-100, and the enzyme fractions to be tested. Activity tests with RT p66 and RT p66/p51 were done in the presence of 80 mM KCl, whereas a concentration of 10 mM KCl was used for RT p51 (for the rationale, see reference 34). The DNA-dependent DNA polymerase activity was determined under the same conditions but in the presence of 0.5 μg of poly(dA)-$oligo(dT)_{15–18}$ or in the presence of 100 ng of single-stranded primed M13 DNA and 40 μM dATP, dCTP, and dGTP, respectively and [^{3}H]dTTP as described above (12). DNA polymerase α was tested with activated calf thymus DNA as described in reference 36. One unit of DNA polymerase activity corresponds to the incorporation of 1 nmol of deoxynucleoside monophosphate into acid-precipitable material in 60 min at 37°C.

(ii) Strand displacement DNA synthesis. To measure strand displacement DNA synthesis, the following components were used in a final volume of 25 μl: 50 mM Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 80 mM KCl; 0.2 mg of bovine serum albumin per ml; 1 mM dithiothreitol; dATP, dGTP, and dTTP (each at 30 μM); 1 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol); 75 ng of the circular, gapped DNA substrate; and various amounts of RT as indicated. The mixture was incubated for 1 h at 37°C unless otherwise noted. Samples were heated at 65°C for 10 min to inactivate the enzymes, and the DNA was then digested after addition of *Hin*dIII (1 U) at 37°C for 30 min. Finally, a precipitation with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate was carried out, and the washed and dried pellets were resuspended in 20 μl of buffer E. The DNA samples were heated for 5 min at 95°C before being loaded onto a 10% polyacrylamide gel containing 7 M urea (17 cm by 21 cm by 0.8 mm). Electrophoresis was performed at 30 V/cm until the bromphenol blue dye reached the bottom. The gel was finally fixed in 10% acetic acid containing 12% methanol, dried at 80°C, and exposed to X-ray film (Kodak X-Omat S).

PhosphorImager. Gels with labelled separated DNA products were exposed for 1 h to a special screen for a PhosphorImager (Molecular Dynamics). After exposure, the screen was scanned into the PhosphorImager, and the indicated regions (see the respective figures) were quantified by using the Image Quant program of Molecular Dynamics.

Other methods. Protein determinations were performed according to the method in reference 7, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method in reference 19.

Nucleotide sequence accession number. Sequences were obtained from GenBank under the following accession numbers: FIV Petaluma, M25729; HIV-1 BH10, M15654; and Moloney murine leukemia virus (MMLV), J02255.

RESULTS

Cloning of the p66 and p51 FIV RT subunits. The p51 and the p66 subunits of FIV RT were separately cloned into pGEX-2T to be expressed as GST fusion proteins. Sequence homology with the HIV-1 RT was used to find the putative cleavage sites of the FIV protease in the *pol* precursor polyprotein (10). Cleavage sites were found to be after Trp-442 for p51 and after Gly-559 for p66. In FIV Petaluma, this corresponds to nt 2336 to 3661 for the p51 sequence and nt 2336 to 4012 for the p66 sequence (32).

Genomic DNA of FL-4 lymphocytic cells permanently infected with FIV Petaluma (40) was used to amplify the two RT subunits by PCR. Amplification primers contained add-on sequences for the restriction endonucleases *Eco*RI and *Cla*I at the 5′ site and *Eco*RI and *Xho*I at the 3′ site. To express these proteins in *E. coli* without the help of the FIV processing protease, a stop codon was also placed immediately after the codon corresponding to the proteolytic cleavage site. The amplification products were cut with *Eco*RI and introduced into the *Eco*RI site of the modified expression plasmid pGEX-2T, a GST fusion expression vector containing a thrombin cleavage site and a labelling site for bovine HMK (3), thus generating the GST-RT p51 expression vector pMA123 and the GST-RT p66 expression vector pMA127 (Fig. 1A).

Expression and purification of both FIV RT subunits. Proteins were purified from IPTG-induced JM109 cells containing either pMA123 or pMA127 by using GSH beads. The GST-RT fusion proteins bound to the beads were then cut with thrombin. This led to 20 additional amino acids at the N terminus of the RT (Fig. 1B). The eluate was finally purified over a Mono S column and analyzed for purity on an SDS–10% polyacrylamide gel (Fig. 2). The large amounts of protein bands in lanes 1 and 2 are due to the boiling of the GSH beads. The presence of large amounts of protein bands demonstrates some unspecific binding of non-GST fusion proteins to the beads and the necessity for a further purification step. Cutting the fusion proteins on the beads with thrombin avoided possible elution of the contaminants. The p66 and p51 subunits of the HIV-1 RT (lanes 3 and 4) were expressed and purified with an identical protocol and compared with the p66 and p51 subunits of the FIV RT (lanes 5 and 6). The FIV RT p66 was slightly contaminated with a degradation product around 51 kDa. When protease inhibitors were omitted during purification, a heterodimeric FIV RT p66/p51 form could be isolated from

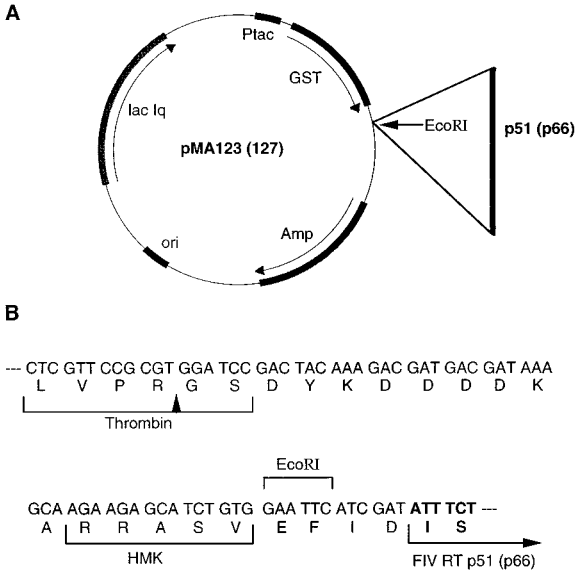

FIG. 1. Map of the expression vectors for the FIV RT p51 and p66 subunits and sequence at the cloning site. (A) Vectors that are derivatives of the protein expression vector pGEX-2T. The PCR-amplified FIV RT p51 and FIV RT p66 gene fragments were cloned into the *Eco*RI site of pGEX-2T as described in the text and in Materials and Methods. They are named pMA123 for FIV RT p51 and pMA127 for FIV RT p66. (B) DNA and amino acid sequences at the cloning site. Amp, ampicillin resistance gene; lac Iq, *lacI*^{q}.

clone pMA127, most likely generated by bacterial proteases (lane 7). Under nondenaturing conditions, the molecular mass of the FIV RT heterodimer was estimated to be around 110 kDa, which confirmed published data (22), while the FIV RT p51 and p66 subunits run slightly faster and slower, respectively, than the heterodimer, suggesting the notion of dimer formation (data not shown).

Functional characterization of both FIV RT subunits. The availability of the two subunits of the FIV RT allowed the separate determination of the optimal salt conditions and comparison with the properties of the HIV-1 RT homodimers. The properties of the homodimers were compared on three different templates (Table 1) as follows. (i) On poly(rA)–oligo $(dT)_{15–18}$, the FIV RT subunits show a higher optimum for $MgCl_2$ than the HIV-1 RT subunits when compared with each other. The FIV RT p66 homodimer requires less KCl than the HIV-1 RT p66 homodimer, whereas FIV RT p51 needs more KCl than HIV-1 RT p51 to perform optimal DNA synthesis. (ii) On poly(dA)-oligo$(dT)_{15–18}$, FIV RT p66 has a reduced requirement for $MgCl_2$ compared with that of HIV-1 RT p66. FIV RT p51 needs no KCl, in contrast with its HIV-1 counterpart. (iii) On single-stranded primed M13 DNA, both FIV RT subunits require 7.5 mM $MgCl_2$ compared with 10 mM for the two HIV-1 RT subunits. In contrast, the latter two need no KCl at their optimum.

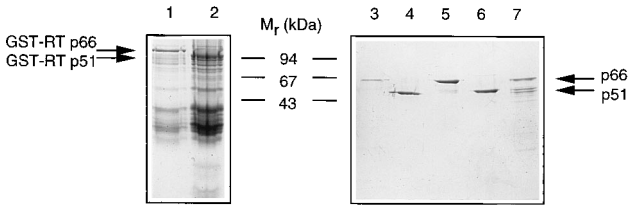

FIG. 2. Expression and purification of the 66- and 51-kDa homodimeric subunits of FIV RT and the FIV RT p66/p51 heterodimer. Enzymes were induced and purified as described in Materials and Methods, and 200 ng was loaded on an SDS 10% polyacrylamide gel. The gel was stained with Coomassie blue. Lanes: 1 and 2, fusion proteins GST RT p66 and GST RT p51, respectively, bound to GSH beads (10 μl); 3 to 7, eluates from the GSH beads, obtained as described in Materials and Methods; 3, HIV-1 RT p66; 4, HIV-1 RT p51; 5, FIV RT p66; 6, FIV RT p51; 7, FIV RT p66/p51 and FIV RT isolated from pMA127 in the absence of protease inhibitors. Degradation products in lanes 1 and 2 are due to the boiling of the beads before loading. Slight degradation products were also seen in lanes 3 and 5 and, especially, lane 7. The markers used were rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), and hen egg white ovalbumin (43 kDa).

TABLE 1. Magnesium and KCl optima of the expressed HIV-1 and FIV RT homodimers[a]

Template and salt	Optimum amt of salt (mM) for enzyme			
	HIV-1 RT p66	HIV-1 RT p51	FIV RT p66	FIV RT p51
Poly(rA)-oligo(dT)				
$MgCl_2$	7.5	2.5	10	5
KCl	120	0	80	10
Poly(dA)-oligo(dT)				
$MgCl_2$	10	7.5	7.5	7.5
KCl	0	40	0	0
Single-stranded primed M13				
$MgCl_2$	10	10	7.5	7.5
KCl	0	0	80	40

[a] The enzyme assays were carried out as described in Materials and Methods.

Furthermore, the specific activities for RNA- and DNA-dependent DNA synthesis were determined for the FIV RT p66/p51 heterodimer, the FIV RT p66 homodimer, and the FIV RT p51 homodimer under the optimal conditions designated by the data in Table 1 (Table 2). The FIV RT p66 homodimer has only a 10% lower specific activity for RNA-dependent DNA synthesis on poly(rA)-oligo$(dT)_{15–18}$ (81,330 U/mg) than the FIV p66/p51 heterodimer (90,030 U/mg), and the specific activity of the FIV p51 homodimer reaches 50% of the level of activity of the heterodimer (45,000 U/mg). The DNA-dependent DNA synthesis is only 4% compared with the RNA-dependent DNA synthesis. On poly(dA)-oligo$(dT)_{15–18}$, the specific activity of FIV RT p66 is 22% (430 U/mg) of the specific activity of the heterodimer (1,950 U/mg). Finally, the specific activity of p51 is extremely low (25 U/mg). On single-stranded primed M13 DNA, the specific activity of FIV RT p66 is 84% (3,880 U/mg) of the specific activity of the heterodimer (4,630 U/mg), while the specific activity of p51 is again very low (76 U/mg). The recombinant forms of FIV RT (p66 homodimer and p66/p51 heterodimer) are equally sensitive to 3′-azido-3′-deoxythymidinetriphosphate compared with the

TABLE 2. Specific activity of genetically engineered FIV RT heterodimer and homodimers

Enzyme	Sp act (U/mg of protein) with template[a]		
	Poly(rA)-oligo(dT)	Poly(dA)-oligo(dT)	Single-stranded primed M13
FIV RT p66/51	90,030	1,950	4,630
FIV RT p66	81,330	430	3,880
FIV RT p51	45,000	25	76

[a] Specific activities were determined as described in Materials and Methods.

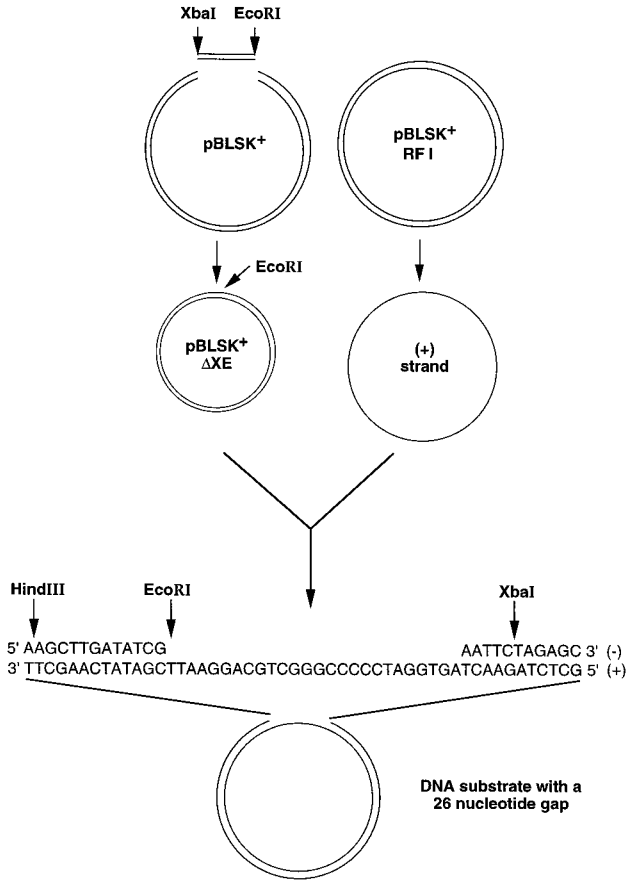

FIG. 3. Scheme for the preparation of a gapped circular double-stranded DNA substrate. Details of the substrate preparation are described in Materials and Methods. Briefly, the *Xba*I-*Eco*RI (XE) fragment in the polylinker site of the circular, double-stranded plasmid Bluescript SK(+) (pBLSK$^+$) was cut out, and the ends of the vector were filled by the Klenow DNA polymerase and religated, thereby restoring the *Eco*RI restriction site. This smaller plasmid was linearized by *Eco*RI digestion and annealed to the circular, positive strand of Bluescript SK, resulting in a circular, double-stranded DNA substrate with a defined single-stranded gap of 26 nt. RF I, DNA replicative form I.

virion-purified FIV RT (29) and genetically expressed HIV-1 RT p66/51 (12) (data not shown).

FIV RT p66/p51 and p66 can perform strand displacement DNA synthesis. To measure strand displacement DNA synthesis as well as the length of the displaced sequence, we used a circular, double-stranded DNA with a defined gap. The steps of DNA substrate synthesis and its final structure are shown in Fig. 3 and are further described in Materials and Methods. From our earlier observation, it was known that the eukaryotic DNA polymerase α is able to fill a gap but has no strand displacement activity (26). Therefore, DNA polymerase α was always included as a control for gap filling (Fig. 4, lane 2). Since we have previously shown that HIV-1 RT p66/p51 possesses strand displacement DNA synthesis activity (12), this enzyme was used as a positive control for strand displacement DNA synthesis (lane 3). For a meaningful comparison, the activities of HIV-1 RT p66/p51 and the different forms of FIV RT were first tested in a standard RT assay with poly(rA)-oligo(dT)$_{15–18}$, and equal amounts (0.1 U) of RT activity were incubated for 30 min with the gapped DNA substrate (leading to a ratio of enzyme to template of 1:1.5). FIV RT p66/p51 could perform strand displacement DNA synthesis almost to the same extent as its HIV-1 counterpart (compare lanes 3 and 4). Both enzymes show major products of equal lengths. To see whether the heterodimer as well as one of its subunits possesses strand displacement DNA synthesis activity, each of the two subunits was tested alone. FIV RT p66 alone could perform strand displacement DNA synthesis (lane 5), whereas no strand displacement products were evident with the FIV RT p51 homodimer (lane 6). The major strand displacement DNA synthesis products of both HIV-1 and FIV RT correspond to 40 and 80 displaced nucleotides (Fig. 4, arrowheads). By prolongation of the incubation time (up to 1 h) or by increasing the amount of enzyme (up to 0.3 U), displacement products of up to 300 nt were obtained (data not shown). Strand displacement was dependent on DNA synthesis, since 3′-azido-3′-deoxythymidinetriphosphate abolished strand displacement (12). No DNA helicase activity could be detected under different conditions, e.g., tested by various NTP concentrations and by comparison with activities of different cellular DNA helicases (35) (data not shown).

FIV RT p51 stimulates strand displacement DNA synthesis of FIV RT p66. Next, we tested the influence of FIV RT p51 on the strand displacement DNA synthesis of FIV RT p66. Figure 5A (lanes 4 to 7) shows different ratios of FIV RT p51 to FIV RT p66. Compared with FIV RT p66 alone (lane 3), a significant enhancement of strand displacement DNA synthesis occurred upon addition of excess FIV RT p51 over FIV RT p66. There was also an increase in the amount of products with a length around 38 nt, suggesting that p51 also enhances the DNA synthesis capacity of p66 on single-stranded DNA (gap filling). FIV RT p51 alone (lane 8) at the highest amount tested has, as already shown in Fig. 4, no DNA synthesis activity. Figure 5B represents the quantification of all bands in lanes 4 to 7 characteristic either of gap filling (region 1) or of strand displacement DNA synthesis (region 2). At a ratio of 5:1 of FIV RT p51 to FIV RT p66, the addition of FIV RT p51 to FIV RT p66 stimulated both gap filling and strand displacement DNA synthesis twofold (Fig. 5B).

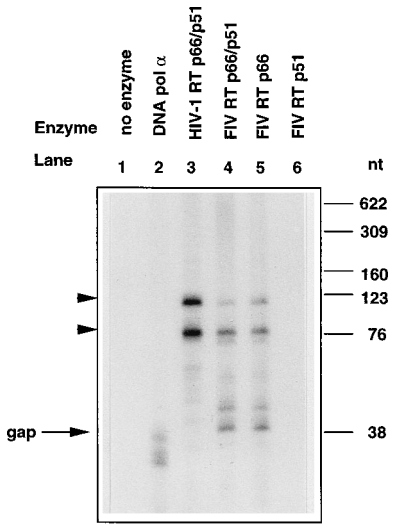

FIG. 4. Strand displacement DNA synthesis activity by three forms of FIV RT and by the HIV-1 RT p66/p51 heterodimer. The strand displacement DNA synthesis assay was carried out as described in Materials and Methods. Lanes: 1, no-enzyme control; 2, DNA polymerase α (pol α), 0.5 U, gap-filling control; 3 to 6, reaction mixtures, each containing 0.1 U of enzyme (16 fmol) defined on poly(rA)-oligo(dT)$_{15–18}$ and 50 ng of template (25 fmol of 3′ OH ends) and each incubated for 30 min. The major products corresponding to 40 and 80 displaced nucleotides are indicated by arrowheads.

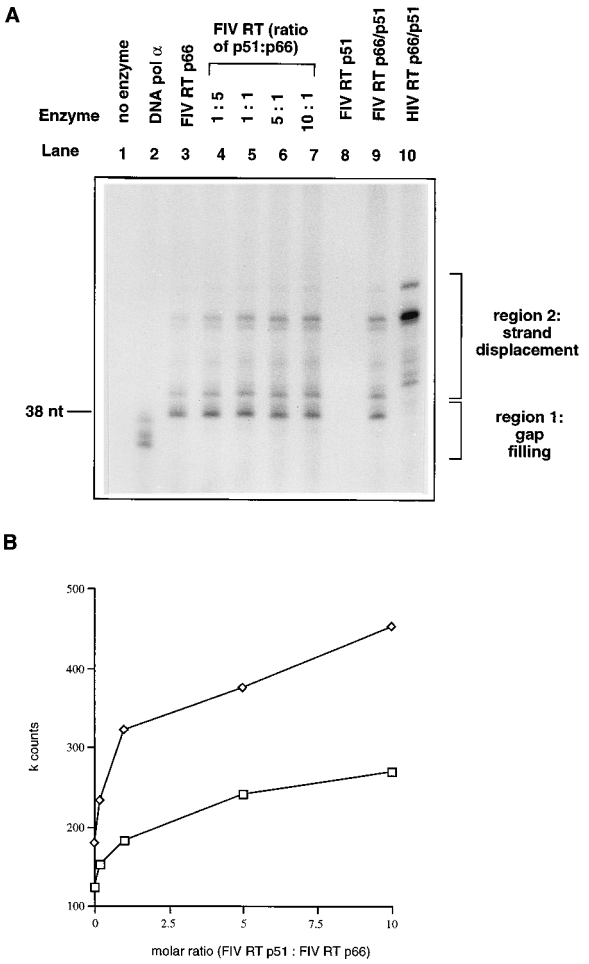

FIG. 5. Strand displacement DNA synthesis activity by FIV RT p66 in presence of increasing amounts of FIV RT p51. (A) Reactions were carried out under conditions described in Materials and Methods. Gap filling was performed with 0.5 U of DNA polymerase α (pol α) (lane 2). Controls were FIV RT p66/p51 (lane 9) and HIV-1 RT p66/p51 (lane 10) (0.1 U each). Lanes 3 to 7 contained 0.1 U of FIV RT p66 and were supplemented with various molar amounts of FIV RT p51 as follows (by lane): 3, none; 4, 1:5 (FIV RT p51 to FIV RT p66); 5, 1:1 (FIV RT p51 to FIV RT p66); 6, 5:1 (FIV RT p51 to FIV RT p66); 7, 10:1 (FIV RT p51 to FIV RT p66); and 8, same amount of FIV RT p51 as was used as in lane 7 but in the absence of FIV RT p66. The two subunits were preincubated for 10 min at 37°C. (B) The two regions, 1 (gap filling [□] and 2 (strand displacement [◇]), were quantified by using a PhosphorImager, and the counts were blotted against the different amounts of FIV RT p51 added to 0.1 U of FIV RT p66.

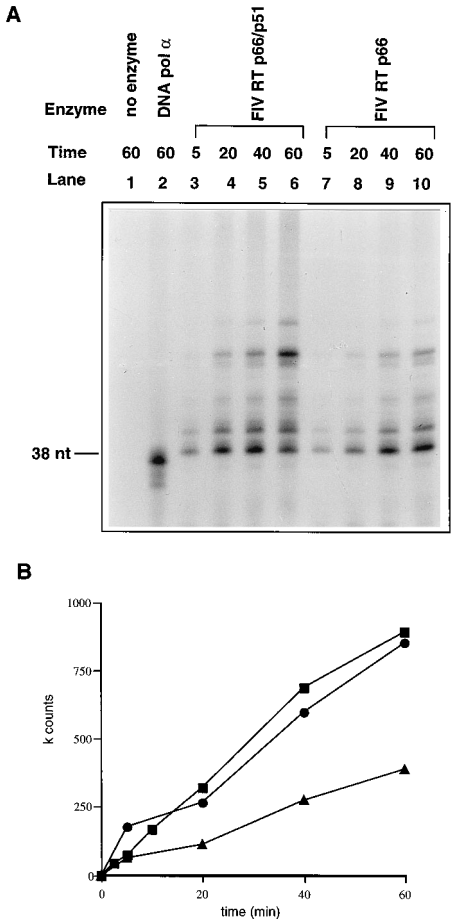

FIG. 6. Kinetics of strand displacement DNA synthesis of FIV RT p66 in the presence and absence of FIV RT p51 and reconstitution of strand displacement DNA synthesis activity of FIV RT p66 by FIV RT p51. Reactions were carried out under conditions described in Materials and Methods. (A) Lanes: 1, no enzyme control; 2, DNA polymerase α (pol α), 0.5 U, 60 min, gap-filling control; 3 to 6, FIV RT p66/p51, 0.1 U; 7 to 10, FIV RT p66, 0.1 U. Reactions were stopped after 5, 20, 40, and 60 min. (B) Three kinetics experiments were run in parallel: FIV RT heterodimer p66/p51 (0.1 U), FIV RT p66 (0.1 U), FIV RT p51, and FIV RT p66 (0.1 U) at a molar ratio of 5:1 (FIV RT p51 to FIV RT p66) that had been preincubated for 10 min at 37°C. Aliquots were removed after 0, 5, 20, 40, and 60 min (additionally after 2.5 and 10 min for the reconstituted heterodimer), separated on a denaturing 10% urea–polyacrylamide gel, dried, and exposed, and the DNA products corresponding to region 2 (strand displacement) indicated in Fig. 5A were quantified by using a PhosphorImager as described in Materials and Methods. ■, reconstituted FIV RT p66 plus p51; ●, FIV RT heterodimer p66/p51; ▲, FIV RT p66.

The rate of strand displacement DNA synthesis can be restored upon addition of FIV RT p51 to FIV RT p66. In a kinetics experiment, strand displacement DNA synthesis products of FIV RT p66/p51 were compared with those of FIV RT p66 at the same time points (Fig. 6A). From the quantification of the band intensities with a PhosphorImager (Fig. 6B), a significant difference in the rate of strand displacement DNA synthesis was observed. The FIV RT p66 strand displacement DNA synthesis could be fully restored with FIV RT p51 at a molar rate of 5:1 (FIV RT p51 to FIV RT p66) in comparison with results with the FIV RT p66/p51 heterodimer (Fig. 6B). These data let us conclude that FIV RT p51 facilitates strand displacement DNA synthesis of FIV RT p66.

The FIV RT heterodimer p66/p51 and FIV RT p66 can catalyze strand displacement in a single round of DNA synthesis. Finally, an enzyme trap experiment (9) was performed to support the suggestion that FIV RT p66/p51 and FIV RT p66 can perform strand displacement in a single round of DNA synthesis. First, the FIV RT forms were incubated with the gapped substrate alone and were compared with FIV RT forms incubated together with gapped substrate and a 500-fold excess of the challenger DNA poly(rA)-oligo$(dT)_{15-18}$. Second, the FIV RT forms were first incubated with the gapped substrate at 37°C, and after 1 min, the 500-fold challenger DNA excess was added. From Fig. 7, it is evident that the FIV RT p66/p51 heterodimer is more able to perform strand displacement DNA synthesis in a single round than is the FIV RT p66 homodimer alone.

DISCUSSION

In order to compare the RTs of HIV-1 and FIV, we have separately cloned and functionally expressed the two subunits

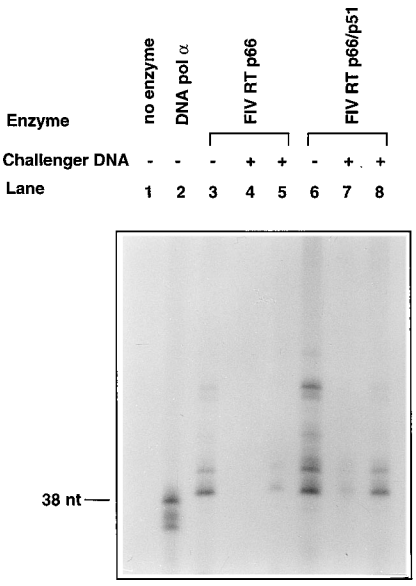

FIG. 7. Heterodimeric FIV RT p66/p51 and FIV RT p66 catalyze strand displacement DNA synthesis in a single round in vitro. Reactions were carried out under conditions described in Materials and Methods. Lanes: 1, no-enzyme control; 2, DNA polymerase α (pol α) 0.5 U, gap-filling control; 3, FIV RT p66 (0.1 U), which was first mixed with the substrate (26 fmol of 3′ OH ends) for 2 min at 37°C, followed by the addition of the dNTP mixture containing [α-^{32}P]dCTP; 4, FIV RT p66, which was mixed with the substrate and a challenger DNA, poly(rA)-oligo(dT)$_{15–18}$ (20 pmol of 3′ OH ends, 500-fold excess), for 2 min at 37°C, followed by the addition of the dNTP mixture; 5, FIV RT p66, which was first mixed with the substrate for 2 min at 37°C, followed first by addition of the dNTP mixture with subsequent incubation for 1 min at 37°C and second by addition of a challenger DNA, poly(rA)-oligo(dT)$_{15–18}$; 6 to 8, as for lanes 4 to 6 but with the FIV RT p66/p51 heterodimer (0.1 U) instead of FIV RT p66.

of FIV RT. Both subunits are enzymatically active; their activities are similar to those of the HIV-1 RT forms. The results furthermore indicated that the FIV RT heterodimer p66/p51 can perform strand displacement DNA synthesis. FIV RT p51 alone was active on synthetic poly(rA)-oligo(dT) but could not perform extensive DNA-dependent DNA synthesis on single-stranded primed M13 DNA. On the other hand, DNA synthesis on both of these two templates was demonstrated with FIV RT p66. In addition, FIV RT p66 was able to perform strand displacement DNA synthesis, although to a lesser extent than the FIV RT p66/p51 heterodimer. However, the activity of FIV RT p66 was enhanced upon addition of FIV RT p51. The enhancement of strand displacement DNA synthesis activity could already be seen at a molar ratio of 1:5 (FIV RT p51 to FIV RT p66). At a molar ratio of 5:1, the rate and extent of strand displacement DNA synthesis could completely be restored. These results are comparable to those found previously for HIV-1 RT (12). This underlines the similarity of the HIV-1 and FIV RTs and helps establish FIV infection as a good animal model for human AIDS. Unfortunately, the crystal structure of the FIV RT has not yet been determined. However, the recent demonstration of the high level of structural similarity between the FIV and HIV-1 proteinases (39) makes it most likely that the three-dimensional structure of FIV RT is very similar to the crystal structure of HIV-1 RT (17), since the similarity between the RTs is even higher than that between the two proteinases.

Overall, our data strengthen the suggestion that one function of the small subunit p51 in FIV RT and HIV-1 RT is to enhance the rate and extent of DNA synthesis and strand displacement DNA synthesis activity of the large subunit p66. Until now, no other catalytic function of FIV RT p51 or HIV-1 RT p51 could be found. The strand displacement activity of HIV-1 RT and FIV RT heterodimer p66/p51 could play a critical role in the replication cycle (6). For the completion of the LTRs and the generation of linear double-stranded viral genomic DNA, it is conceivable that a strand displacement DNA synthesis activity might be required in later stages of lentiviral reverse transcription.

The fact that displaced products up to 300 bases in length could be found indicated that FIV RT and HIV-1 RT are able to perform productive strand displacement DNA synthesis. However, we found that the lengths of the observed fragments were dependent on the enzyme/template ratio and the incubation time. For MMLV, RT strand displacement DNA synthesis products longer than 1 kb were demonstrated (38). In these experiments, however, a large excess of enzyme (200 U; 70-fold molar excess) and a 3-h incubation were used. Taken together, these results suggest that FIV RT and HIV-1 RT are able to perform strand displacement DNA synthesis at the LTRs alone, but participation of cellular or other viral factors (e.g., helicases, nucleocapsid protein) cannot be excluded. Strand displacement DNA synthesis was demonstrated for FIV RT, HIV-1 RT (12), MMLV RT (38), Φ29 (4), and bovine DNA polymerase δ (26) (Table 3). When the amino acid sequences of these polymerases were compared, the motif Y-X_3-D-X_7-L, common in all enzymes that can perform strand displacement DNA synthesis, could be identified (Fig. 8). No strand displacement DNA synthesis was found for all DNA polymerases α (26), human DNA polymerase ε (16, 26), herpes simplex virus DNA polymerase (18), and *E. coli* DNA polymerases II

TABLE 3. Correlation between strand displacement DNA synthesis activity and the proposed strand displacement motif[a]

DNA[b]	Strand displacement	Motif
HIV-1 RT	+	+
FIV RT	+	+
MMLV RT	+	+
Φ29 polymerase	+	+
Polymerase δ		
Bovine	+	+
Human	NT[c]	+
Mouse	NT	+
T7 polymerase	NT	+
Epstein-Barr virus polymerase	NT	+
M2 polymerase	NT	+
Human polymerase ε	−	−
Polymerase α (all)	−	−
Herpes simplex virus polymerase	−	−
E. coli		
Polymerase III	−	−
Polymerase II	−	−

[a] For details about the motif, see the legend to Fig. 8.
[b] For citations, see text.
[c] NT, not tested.

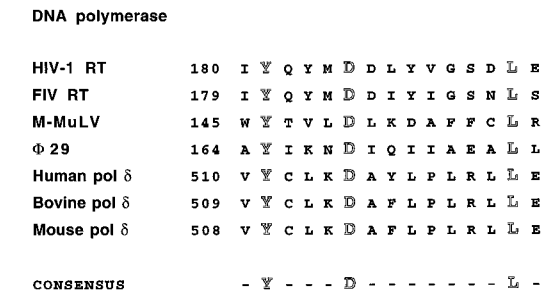

FIG. 8. Amino acid motif identified in DNA polymerases with strand displacement DNA synthesis. For citations see text. M-MuLV, MMLV; pol, polymerase.

and III (18) (Table 3). Interestingly, all of these polymerases do not have the motif in their amino acid sequence. This motif does not necessarily have to be located in the polymerase active site, as outlined for the eukaryotic replicative DNA polymerases δ and for MMLV RT. However, for the two lentiviral RTs, this motif lies in the active site and contains the YXDD box (Fig. 8). Demonstration of strand displacement DNA synthesis in other DNA polymerases with this motif would strengthen our findings. Site-directed mutagenesis experiments will reveal which of the conserved amino acids are involved in strand displacement DNA synthesis.

ACKNOWLEDGMENTS

We thank Hans Lutz (University Veterinary Hospital, Zurich) for donating FL-4 cells.

This work was supported by the Swiss National Science Foundation (AIDS Program 31.37146.93). M.H. is a recipient of the Swiss National Science Foundation Grant for the M.D.-Ph.D. Program (3135-36713.92).

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